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Title: Superior efficacy of an HIV vaccine combined with ARV prevention in SHIV challenged non-human primates

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Running Title: Superior Efficacy of Combined Vaccine and Microbicide in NHP

Key Words: Microbicide, Vaccine, combination prevention, ART, HIV, prophylaxis

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ABSTRACT:

Although vaccines and antiretroviral (ARV) prevention have demonstrated partial success against HIV infection in clinical trials, their combined introduction could provide more potent protection. Furthermore, combination approaches could ameliorate potential increased risk of infection following vaccination in the absence of protective immunity. We used a non-human primate model to determine potential interactions of combining a partially effective ARV-microbicide with an envelope-based vaccine. The vaccine alone provided no protection from infection following 12 consecutive low dose intravaginal challenges with SHIVSF162P3, with more animals infected compared to naïve controls. The microbicide alone provided a 68% reduction in risk of infection relative to the vaccine group and a 45% reduction relative to naïve controls. The vaccine-microbicide combination provided an 88% reduction in per exposure risk of infection relative to vaccine alone, 79% reduction relative to controls. Protected animals in the vaccine-microbicide group were challenged a further 12 times in the absence of microbicide and demonstrated a 98% reduction in risk of infection. Taken together a total risk reduction of 91% was observed in this group over 24 exposures (P=0.004). These important findings suggest that combined implementation of new biomedical prevention strategies may provide significant gains in HIV prevention.

IMPORTANCE:

There is a pressing need to maximize the impact of new biomedical prevention tools in the face of 2 million HIV infections that occur each year. Combined implementation of complementary biomedical approaches could create additive or synergistic effects that drive improved reduction in HIV incidence. Therefore, we assessed combining an untested vaccine with an anti-retroviral (ARV) based microbicide in a non-human primate vaginal challenge model. Vaccine alone provided no protection (and may have increased susceptibility to SHIV challenge vaginal), while the microbicide reduced infection risk compared to vaccinated and naïve animals. Importantly, the combined interventions provided the greatest level of protection which was sustained following withdrawal of the microbicide. The data suggests provision of ARV prophylaxis during vaccination reduces the potential for unexpected increased risks of infection following immunization and augments vaccine efficacy. These findings are important for potential adoption of ARV-prophylaxis as the baseline intervention for future HIV/AIDS vaccines.

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INTRODUCTION:

The Thai RV144 vaccine trial, based on a canary-pox vector prime (ALVAC)–protein boost (AIDSVAX), is the first clinical trial to have shown moderate efficacy (31.2%) in cohorts at low risk of HIV exposure [1]. Partial protection has also been observed with other new biomedical approaches including the use of antiretroviral (ARV) drugs as oral (44-75%) or topical (vaginal, 39%) pre-exposure prophylaxis (PrEP) [2-5]. However, effectiveness was dependent upon consistent product use and impacted by multiple factors influencing susceptibility and exposure risk [6]. Three decades of research on combined implementation of structural and behavioural interventions have indicated that combination approaches are more effective than any single intervention alone [7]. Additional potential gains could be realised by assessing the impact of combining new biomedical prevention strategies [8]. Indeed, positive impact would be seen if combining ARV prevention and vaccines provides better protection than either intervention alone. Here, reduction of the number of transmitted strains and/or delay in the initial viral expansion phase might buy time for more effective immune clearance. Conversely systemic immunity might curtail dissemination of virus that bypasses the activity of topically applied ARVs. Furthermore, subjects protected from productive infection on repeat exposure to HIV when using ARV-prevention, might evoke exposure-induced immunity. This could serve to modify vaccine-induced immune responses to better recognize prevalent circulating virus. Indeed, evidence from some non-human primate (NHP) studies indicates that animals exposed to infectious virus when protected by PrEP demonstrate cellular immune responses to the challenge virus [9, 10]. However, such immune responses in these non-vaccinated animals appeared insufficient to protect animals from subsequent challenge in the absence of PrEP [10]. Conversely, combinations could also have potential negative interactions. Certain vaccine induced immune activation may have potential to increase mucosal HIV-1 susceptibility [11, 12]

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that in combination could reduce the efficacy of ARV-prevention. This has important implications given that increased sensitivity over the potential of novel vaccines to enhance the risk of HIV acquisition may drive the adoption of oral pre-exposure prophylaxis provision as the baseline intervention for future HIV/AIDS vaccine trials.

MATERIALS AND METHODS:

Ethics Statement

All 50 Mauritius-origin, outbred, young adult (4-6 years old) female cynomolgus monkeys (*Macaca fascicularis*) were housed in the CEA facilities ("Commissariat a l'Energie Atomique", Fontenay-aux-Roses, France, CEA accreditation no.: B 92-032-02) in compliance with Standards for Human Care and Use of Laboratory of the Office for Laboratory Animal Welfare (OLAW, USA). The study and procedures were approved by ethical committee "Comité Régional d'Ethique pour l'Expérimentation Animale Ile-De-France Sud" with notification number 10-062. All experimental procedures were carried out in the CEA animal facility and in strict accordance with European guidelines for NHP care (European directive 86/609, then, as for January 2013, EU Directive N 2010/63/EU) for protection of animal used in experimentation and other scientific purposes and the Weatherall Report. The monitoring of the animals was under supervision of veterinarians in charge of the animal facilities. All efforts were made to minimize suffering, including improved housing conditions with enrichment opportunities (e.g. 12:12 light dark scheduling, provision of treats as biscuits and supplemented with fresh fruit, constant access to water supply in addition to regular play interaction with staff caregivers and research staff). Experimental procedures were performed while animals were under anesthesia using 10mg/kg (body weight) of ketamine. Euthanasia was performed prior to development of symptoms of disease (indicated by a rapid decline in $CD4^+$ T cells and or increase in viremia) and was

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performed by IV injection of a lethal dose of pentobarbital. All 50 animals described were experimentally naïve at the beginning of the study. Investigators were blind to the group allocation while performing immunological and virological assessments.

Cynomologus macaque combined micorobicide, vaccine study

Tenofovir gel (1%) or control placebo gel, a proprietary formulation containing purified water with edetate disodium, citric acid, glycerin, methylparaben, propylparaben, and hydroxyethylcellulose (pH 4.5) provided by CONRAD, (Arlington, VA) was transferred to 5-ml syringes and administered in 2-ml volumes via a 10 FG soft catheter introduced ~2 cm into the vagina. The process was atraumatic with no obvious leakage and carried out while the animals were under anesthesia. Vaccine antigens, uncleaved gp140 TV1 and SF162, and MF59 adjuvant were provided and manufactured by Novartis. For each intranasal (IN) immunization, 50µg each of TV-1 and SF162 gp140 was given in solution in a volume of 0.2ml containing 500ug of Resiquamod (R848) a TLR 7/8 agonist (Invivogen). The solution was dropped into each anterior nares of sedated animals placed in a prone position with their heads tilted back. For intramuscular (IM) immunizations 100µg each of TV-1 and SF162 gp140 was mixed with MF59 adjuvant and given in a volume of 0.4mls into the deltoid muscle of the upper arm. Vaccinated cynomolgus macaques received three IN priming immunizations (0, 4, 8 weeks) followed by two IM boosting immunizations (16 and 28 weeks). Challenge studies were commenced 11 weeks after the final boost immunization.

50 Mauritius-origin, outbred, young adult (4-6 years old), female cynomolgus monkeys (*Macaca fascicularis*) were utilized for this study. No randomization was used however groups were balanced for susceptible and resistant MHC haplotypes (H2, H6 and H4) [13]. Recent studies demonstrated that TRIM5a genotype has no impact on virus acquisition or vaccination

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outcome [14]. Sample size was chosen knowing that 90% infection was expected in control group based on previous titration of the challenge stock. A sample size of 16 for each of the M and V+M groups was chosen with an 80% power to detect an increase in survival proportion of 0.53 with a significance level (alpha) of 0.05 (two-tailed log rank test). A sample size of 12 animals for the controls provided an 80% power to detect an efficacy of 67% in the other groups. Only 8 animals available for inclusion in group V however this number was estimated sufficient to prove that vaccine alone was ineffective in preventing infection. Investigators performing the animal studies were not blinded as to group allocation. On the day of challenge, ~2-mL of the microbicide gel, was applied atraumatically to the vagina (M and V+M groups), 1h before viral challenge. $SHIV_{162P3}$ was added in a 1-mL volume containing 0.5 AID_{50} of *in vivo* titrated stock of the R5 virus $SHIV_{162P3}$ [15], derived from the HIV-1 SF162 primary isolate and propagated in phytohemagglutin (PHA)-activated rhesus macaque peripheral blood mononuclear cells (PBMC). Stock was obtained through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH (cat. no. 6526; contributors: Janet Harouse, Cecilia Cheng-Mayer, and Ranajit Pal). Monkeys were bled weekly for viral loads, and infection status was determined by measuring plasma viral load using an RT-PCR assay with a sensitivity limit of 60 RNA copies/ mL and a quantification limit of 300 RNA copies/mL [16]. No further vaginal treatments were halted on detection of viremia. Animals were followed to determine set point viral loads. We excluded macaque #25015 from group M because at autopsy 2 months post infection we found a malformation of the genital tract: direct connection between vagina, uterus and peritoneal cavity. We also excluded animal #28413 from M group due to a technical failure to deliver the full dose

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of viral challenge. A schematic representation of the vaccination schedule, vaccination groups and immunizations for part 1 and 2 is given in Fig. 1A.

Immunogenicity antibody analysis

TV-1 and SF162 specific binding antibodies were analyzed in serum and mucosal secretions. Briefly, 96 well plates were coated with a 1:1 ratio of α-Human κ and α-Human λ antibodies (Southern Biotech) to capture the standard curves (IgG or IgA standards) and TV1 and SF162 protein (1µg/ml) to capture antigen specific antibodies. Negative controls consisted of normal cynomologus macaque serum and assay buffer. Standard curves for IgG and IgA consisted of 5fold serial dilutions of purified IgG or IgA (starting at 1µg/ml), macaque serum and mucosal secretions samples were "screened" at 1:100 and 1:10 respectively with samples and controls added in triplicate. Bound IgG was detected with goat anti-monkey IgG (Fc-specific) HRP conjugate (Serotec) and bound IgA was detected with goat anti-monkey IgA (α-chain-specific) HRP conjugate (Autogen Bioclear,). Following secondary antibody addition and development plates were read at 450nm. Positive responses were according to pre-defined cut-off values. Positive samples were titrated and concentrations determined by extrapolation of unknown samples against standards and expressed as µg/ml of specific IgG or IgA.

Peptide array serum specificity mapping

Serum epitope mapping of heterologous strains was performed essentially as previously described [17, 18]. Briefly, a peptide library of overlapping peptides (15-mers overlapping by 12), covering 7 full-length HIV-1 gp160 Env consensus sequences (clades A, B, C, and D, group M, CRF1, and CRF2) and 6 vaccine and laboratory strain gp120 sequences (A244_1, TH023_1, MN_B, 1086_C, TV1_C, and ZM651_C), was printed onto epoxy glass slides (provided by JPT Peptide Technologies GmbH [Germany]). Microarray binding was performed using the HS4800

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Pro Hybridization Station (Tecan, Männedorf, Switzerland). All arrays were blocked with Superblock T20 PBS blocking buffer for 0.5 hour at 30°C, followed by a 2 hr incubation at 30°C with heat inactivated plasma diluted 1:250 in Superblock T20. Arrays were incubated for 45 minutes at 30°C with Goat Anti-Hu IgG conjugated with DyLight649 (Cat #109-495-098, Jackson ImmunoResearch, PA) (1.5 µg/ml final concentration) diluted with Superblock T20. Washes between all steps were with PBS containing 0.1% Tween. Arrays were scanned at a wavelength of 635 nm using an Axon Genepix 4300 Scanner (Molecular Devices, Sunnyvale, CA, USA) at a PMT setting of 540, 100% laser power. Images were analyzed using Genepix Pro 7 software (Molecular Devices). Binding intensity of the post-immunization serum to each peptide was corrected with its own background value, which was defined as the median signal intensity of the prebleed serum for that peptide plus 3 times the standard errors among the 3 subarray replicates present on each slide as described.

Neutralization TZMbl assay analysis

Viral titration and neutralization assays were performed as previously described [19, 20]. The following Pseudotyped viruses (PSV) pCAGGS SF162gp160, BX08, 93MW965.26, TV1.21, TV1.29, QH0692, DJ263.8, pSV7d-SHIVSF162-Qlc32 4014, the infectious molecular clone (IMC) pNL-LucR.T2A-SHIV162P3.5.ecto and the culture supernatant of SHIV162P3 M623-Derived were used. The JC53bl-13 (TZM-bl, Cat No 5011) cell line was obtained from NIBSC Center for AIDS Reagents, UK and validated as mycoplasma free with MycoAlert (Lonza). Four steps of 3-fold dilutions, starting with 1/20 of each serum or mucosal sample, were incubated with virus supernatant (200 TCID50) for 1 hour. Thereafter, 10^4 TZMbl cells were added and plates incubated for 48h, when luciferase activity was measured. Positive controls were sera of HIV-1 infected individuals or macaques and monoclonal antibody known to neutralize the

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viruses. Neutralization titers were defined as the sample dilution at which relative luminescence units (RLU) were reduced by 50% compared to RLU in virus control wells after subtraction of background RLU in control wells with only cells.

ADCC activity

ADCC was tested according to the protocol described in [21] using the IMC pNL-LucR.T2A-SHIV162P3.5.ecto. IMC infected CEM.NKR.CCR5 cells (obtained from cell line was obtained from NIBSC Center for AIDS Reagents (Cat No 0099), UK and validated as mycoplasma free with MycoAlert (Lonza).) were incubated at 1:30 ratio with PBMCs and six four-fold dilutions of each serum starting with 1:100 dilution. The percentage of the cells positive for the GzB substrate are reported as percentage of Granzyme B activity. Positive control was the monoclonal antibody b12.

INF-γ and IL-2 T cell ELISpot assay

ELISpot assays were performed using multiScreen 96-well filtration plates (Millipore, Guyancourt, France) coated overnight at 4°C with monoclonal Ab against monkey IFN-γ (clone GZ-4, Mabtech, Nacka, Sweden) and IL-2 (CT-611 kit, U-Cytech biosciences, Utrecht, the Netherlands) following the manufacturer's instructions. Plates were washed 5 times with PBS then blocked by incubation for 1h at 37°C with RPMI 1640 medium containing glutamax-1 (Gibco, Life technologies, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS, Lonza; culture medium). Fresh isolated PBMC ($2x10^5$ cells per well) were stimulated in duplicate with 2 µg/ml of HIV-1 gp120 SF162 recombinant protein (Novartis, batch N° MID167d) or with SIVmac251 Gag peptide pools (15 mer overlapping of 11 aa). Control wells (10^3 PBMC) were stimulated with medium alone or with PMA/neomycin (1µg/ml). Plates were incubated for 24h (gp120 glycoprotein) or 18h (Gag peptide pools) at 37°C in 5% CO2

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atmosphere. They were then washed 5 times with PBS. Biotinylated anti-IFNγ (clone 7-B6-1, Mabtech) or anti-IL-2 (CT-611 kit, U-Cytech biosciences) Ab were then added at a concentration of 1 µg/ml in 0.5% FCS in PBS and the plates were incubated overnight at 4°C. Plates were then washed 5 times with PBS, incubated with 0.25 µg/ml alkaline phosphatase-streptavidin conjugate (Sigma-Aldrich, St-Quentin Fallavier, France) for 1h at 37°C, washed 5 times with PBS. Spots were developed by adding NBT/BCIP substrate (Sigma-Aldrich) and counted using an Automated Elispot Reader ELR04 XL (Autoimmun Diagnostika GmbH, Strassberg, Germany).

Statistical analyses

A time-to-event analysis was conducted with first Kaplan Meir estimates with log-rank test comparing groups for datasets in Phase A and dataset in Phase B. Then, we used a logistic model with random effects taking into account repeated measures in each macaque. This type of model

$$logit\,P\,infection=1=\alpha 0+\alpha 1t+\alpha 2G$$

allows taking into account the discrete exposure to the infection due to the challenges at given times. Preliminary analyses showed that the variance of the random effect was not significantly different from zero (p=0.25), indicating a low variability of response between monkeys. The first analysis of interest was: where *t* represents the time since vaccination and G represents the group of treatment. The time of infection was taken as the date of previous challenge prior to first positive SIV test. However, sensitivity analysis, taking time of infection as previous challenge or ante-previous challenge did not change qualitatively the results (results not shown).

$$logit\,P\,infection\,=1=\alpha 0+\alpha 1t+\alpha 2V+\alpha 3M+\alpha 4V*M$$

The rationale of including *t* in the regression is to take into account any residual confounding associated to a change of the probability of infection over time. This could be due to the selection

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of the population, those being the most resistant being uninfected until the end. This analysis was run on the dataset of Part 1 and Part 2. Then, we extended the analysis by dissociating the effect of vaccination (V) and microbicide (M) and their interaction (V*M):

Second order interaction between M, V and time were tested but not significant (p=0.91).

However, in a final analysis:

$$logit\,P\,infection=1=\alpha 0+\alpha 1TP2+\alpha 2V+\alpha 3M+\alpha 4V*TP2+\alpha 5V*M$$

Time *t,* was categorized into an indicator of Part 1 or 2 T_{P2}=I (t>22weeks) and the effect of the interaction V* T_{P2} was kept (p=0.22). Results were used to compare effect of V+M in Part 1 versus Part 2. This analysis was run on the dataset in Part 1 and 2 pooled. All results are reported in term of Odd-Ratio (OR), together with their 95% confidence interval and p-values for significance. Results were presented as % of risk reduction but readers should keep in mind that OR are only approximation of risk estimates. We compared the results of logistic regression with the time-to-event Cox model analysis: Hazard ratios (HR) and OR give similar conclusions (results not shown). Analyses were run using R software and packages "survival" for survival and "lme4" for logistic mixed effects models.

RESULTS:

Study design

In this study we use a NHP model to determine potential interactions of combining a microbicide with an envelope-based vaccine over either intervention alone. We chose to study vaginal transmission as most vaccine and microbicide efficacy trials will likely be dependent upon the use of trial sites in sub-Saharan Africa where infection rates are highest among women [22]. We chose to evaluate 1% tenofovir microbicide gel, based on reported efficacy from the CAPRISA 004 trial [5]. We focused on an HIV-1 envelope based vaccine reflecting the likely protective role of antibody in RV144, and adopted a vaccine strategy previously shown to protect NHP

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against vaginal challenge with Tier-1 $SHIV_{SF162p4}$ [23]. We designed a two-part study to test the potential interactions (positive or negative) between these two biomedical strategies over single interventions. In Part 1 we compare the protective efficacy of the envelope based vaccine (V), 1% microbicide tenofovir gel alone (M), and their combination (V+M) against 12 repeat vaginal challenges with the Tier-2 $SHIV_{SF162p3}$ (Fig.1A). Critical to the experimental design was that neither of the individual interventions could be fully protective by themselves, therefore we elected to use $SHIV_{SF162p3}$ over the more closely matched $SHIV_{SF162p4}$, where vaccination was previously shown to provide 100% protection [23]. In Part 2, protected animals were challenged a further 12 times in the absence of microbicide.

Vaccination and vaginal SHIV challenge

Cynomolgus macaques in the vaccine groups (V, and V+M groups) received three intranasal priming immunizations (0, 4, 8 weeks) with a combination of two gp140 uncleaved trimers (TV-1 clade C + SF162 clade B) co-administered with R848 (TLR 7/8) adjuvant, followed by two intramuscular boosting immunizations (16 and 28 weeks) delivered with MF59 adjuvant. This induced robust serum binding antibody responses (IgG and IgA) to both immunogens (TV-1 and SF162 gp140) that remained stable through to week 39 the start of challenge (Fig.1B-E). Low vaginal responses were observed in some animals, post intranasal priming and were boosted following intramuscular immunizations (Fig. 2). Peptide array analysis demonstrated all animals developed a strong cross-clade anti-V3 response, and responses against the gp41 immunodominant region (gp41 ID). Animals also developed cross-clade responses of lower intensity against V2, C2, and C5 gp120 epitopes (Fig. 3). Autologous serum neutralizing antibodies (NAbs) against HIV-1 SF162 were induced following intramuscular boosts (mean 7891±11728 SD) that decreased by approximately 1 log prior to challenge (Fig. 4A).

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Neutralizing antibodies were absent or below the level of detection in vaginal secretions. Serum neutralizing responses were also induced to clade C MW965.26 (mean 1757±2240 SD) but not TV1 or Tier-2 viruses of other subtype week 30 (Fig. 4 B & D). As similar responses had been fully protective against Tier-1 $SHIV_{SF162p4}$ [23] we elected to use the variant $SHIV_{SF162p3}$ that differs by 22 amino acids and contains an additional glycan at the N-terminal base of the V2 loop predicted to confer escape from autologous neutralization [24]. Pre-challenge sera and vaginal samples were confirmed to have little or no neutralizing activity against $SHIV_{SF162p3}$ (Fig. 4C). As predicted based on neutralization, the vaccine alone (group V) showed no protection against 12 consecutive low dose intravaginal challenges with $SHIV_{SF162P3}$ (Fig. 5A and Table 1A), where 50% of the animals in both the V and naïve control group (C) became infected after 2 challenges (Table 2). Strikingly there were more infections with the vaccine than in naïve controls with an odds ratio of 1.73, although this did not reach statistical significance (p=0.341, Table 1A), where the baseline risk of infection was almost double that of controls (Table 2). Furthermore the vaccine alone had no impact on viral load kinetics or control (Fig.5B).

Tenofovir gel and vaginal SHIV challenge

The microbicide regime was designed to be partially protective, in our case application of 1% tenofovir gel applied vaginally 1 hour before each of 12 sequential vaginal challenges. We confirmed in cynomolgus macaques that TDF levels measured in peripheral blood and genital tissue at different time points following 1% Tenofovir gel application reached concentrations compatible with local antiviral activity (Table 3). We also measured TDF-DP in genital tissues as a means to quantify active phosphorylated drug by local exposed cells. In both cases, similar levels to those reported in rhesus macaques [25] and in humans in the CAPRISA 004 trial were observed [26]. To limit the number of animals included in this study (50 in total), we assumed

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the risk of not detecting a benefit of the partially effective microbicide alone (group M) relative to naïve animals (group C). This was indeed the case when compared to naïve controls (OR 0.55, p=0.263, Table 1A). However, based on the Kaplan-Meier curves (Fig. 5A) 9.5 challenges would be required to infect 50% of animals in group M compared to 2 challenges for groups C and V (Table 2). The microbicide alone provided a 68% reduction in risk of infection relative to the vaccine group (OR 0.32, p=0.045, Table 1B). There was however, no apparent impact of tenofovir gel on viral load kinetics in those animals that became infected (Fig. 5B).

Vaccine-microbicide combination provides enhanced protection

As the primary objective was to assess the potential benefit of combining the vaginal microbicide approach in previously vaccinated animals, the study was designed to detect efficacy of the combination (group V+M, n=16) over either intervention alone. For the V+M group animals were vaccinated in parallel to the vaccine only group (V), and challenge studies commenced at week 39. In these animals, the microbicide was applied in an identical fashion to group M for each of the 12 challenges in Part 1 Fig. 1A. The V+M combination provided a 79% reduction in per-exposure probability of infection (p=0.013, Table 1A) relative to naïve controls, an 88% reduction (OR 0.12, p=0.001, Table 1B), relative to vaccine alone, and a 63% reduction (OR 0.39) relative to microbicide alone, although this did not reach statistical significance (p=0.114) (Fig. 5A). Only 4 animals were infected after 12 repetitive challenges, insufficient to predict the number of challenges to reach 50% infection in the M+V group. Animals that remained uninfected following 12 consecutive intravaginal challenges in presence of microbicide (challenge Part 1) immediately progressed to challenge Part 2 (Fig. 1A). Here all protected animals received a further 12 sequential challenges, irrespective of initial assignment to M (n=6) or M+V (n=12) groups, to determine susceptibility in the absence of microbicide. By the end of

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Part 2 (Fig. 5A) there was still no statistical difference between group M and untreated controls in Part 1 (Table 1C), while for the V+M group there was a 84% reduction in per exposure probability of infection (p=0.010) relative to untreated controls and a 86% reduction in per exposure probability of infection relative to vaccine alone (p=0.002). Here the positive interaction between microbicide and vaccine remained the same (interaction coefficient p=0.13). In order to gain further insight, we discretized the time into parts 1 and 2 and re-ran the analysis. This analysis indicated that the microbicide alone over the entire course of parts 1 and 2 showed a trend toward protection [OR 0.26, (0.06:1.02) p=0.054, Table 1D] whereas microbicide and vaccine combined provided significant protection with a 91% reduction in the per exposure probability of infection (p=0.004, Table 1D). Further analysis was performed to investigate the potential interaction between time and vaccination in the V+M group (discretized in Part 1 and 2). The effect of the V+M group compared to control increased in Part 2 providing a 98% (p=0.002) reduction in per exposure probability of infection in Part 2 compared to 89% (p=0.010) in Part 1, indicating a long term effect of vaccination even without microbicide (Table 1E). We excluded potential confounders that might have influenced differences in susceptibility. Distribution of the MHC genotype was equal across the different groups [13]. Furthermore, recent studies demonstrated that TRIM5α genotype has very little variability in Mauritian cynomolgus macaques and has no impact on virus acquisition or vaccination outcome [14]. To more faithfully replicate the human condition animals were not treated with Depo-provera often used to enhance susceptibility of infection. All animals were naturally cycling. Analysis of progesterone levels showed no over representation in any group of animals in the follicular phase, associated with heightened susceptibility.

Immune parameters modulated by protected exposure to infectious SHIV

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Subsequently we assessed potential immune parameters that might be associated with enhanced protection in the V+M group relative to the V or M groups. Prior to challenge serum or mucosal antibody titre in the V and V+M group were similar although serum antibody titres were slightly raised for the V only group ($p=0.0045$, Fig.6A & Fig.1B-D). Induced serum binding antibodies to SF162 and TV-1 gp140 were high pre-challenge and may have masked any potential boosting effects of protected exposure in the V+M group (Fig. 1B). However there was no evidence of boosting in the mucosal samples of protected animals over time. There was little or no neutralization in sera and mucosal samples to $SHIV_{SF162P3}$ prior to challenge in both V and V+M groups (Fig. 4) and no evidence of induced response in protected animals at any point during Parts 1 & 2. However, autologous neutralizing responses to HIV-1_{SF162} were equivalent in the V+M group relative to the V group prior to challenge and post immunizations (Fig. 7A) with no evidence of boosting in protected animals after 6, 9 or 12 challenges in Part 1 (Fig. B-E) or change in epitope recognition assessed by peptide array analysis (data not shown). In addition, there was no evidence of boosting neutralizing responses to HIV-1_{SF162} or $_{MW965}$ in protected animals in Part 1 in the presence of microbicide or Part 2 in the absence of microbicide (Fig. 8). Furthermore, ADCC responses against $SHIV_{SF162P3}$ were absent in all uninfected animals at all-time points.

Robust cellular immune responses against SF162 gp120, were detected by ELISPOT in V and V+M groups after the five immunizations. Responses were similar in both groups Fig. 9 with mean numbers of spot forming cells (SPC) per million PBMC of 369±246 and 274±171 for IFN-γ and IL-2, respectively (Fig. 9A & B). There was no correlation between pre-challenge vaccine induced IFN-γ and IL-2 gp120 specific responses and protection observed after challenges in Parts 1 and 2. We also measured T-cell responses to gp120 stimulation after the first sequence of

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challenges. These had significantly decreased in group V, when compared to pre-challenge measures at week 34 ($p=0.0058$ and $p=0.0002$ for IFN-γ and IL-2, respectively, Fig. 9C & D), down to the level of control animals (group C). Repeated exposure of non-vaccinated animals in group M were similar ($p=0.7315$ and $p=0.9027$ for IFN-γ and IL-2, respectively) to those observed in control animals (group C) indicating no added benefit for naïve animals when exposed to the virus in presence of microbicide.

Remarkably, responses in vaccinated animals appeared to be significantly increased when exposed to $SHIV_{SF162P3}$ following treatment with TDF gel (group V+M) by comparison to animals of group V ($p=0.0058$ and $p=0.0002$ for IFN-γ and IL-2) and to animals of group M ($p<0.0001$ and $p<0.0001$ for IFN-γ and IL-2), demonstrating that repeated challenges in pre-immunized animals when using microbicides for prevention evoke exposure-induced immunity. However, responses raised in protected animals in this group were similar to non-protected macaques. All animals infected in Parts 1 or 2 demonstrated robust responses to SIV Gag peptide pools irrespective of intervention group (Fig. 9G & H), however there were no detectable anti-Gag responses in any of the protected animals following sequential challenges in Parts 1 or 2, irrespective of the intervention group (M or V+M).

DISCUSSION:

The primary aim of this study was to determine any potential benefit from a microbicide and vaccine combination over single prevention approaches. The lack of protection by the vaccine alone, and lack of virologic control when challenged with a Tier-2 autologous escape variant $SHIV_{SF162p3}$ is unsurprising. This contrasts with the earlier observation of protection against a high dose challenge with the SF162 immunogen matched Tier-1 $SHIV_{SF162p4}$ [23]. These data reflect the dominant role of neutralizing antibodies in sterilizing protection, while the absence of

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induced ADCC activity likely accounts for the lack of impact on virologic control. However the observation that 7/8 animals were infected in the vaccine group versus 4/12 in the controls, although not statistically significant, is concerning given the potential for vaccination to increase risk of acquisition in large cohort studies [11, 12]. Interestingly the observed level of protection (43%) in the M group closely matched that reported for women that were highly compliant with the dosing regime in CAPRISA 004 [5], although in our study with limited number of animals this did not reach statistical significance. Gel alone had no impact on viral kinetics, thus at the systemic level there was no evidence to suggest the microbicide delayed or blunted infection. Indeed while protection appeared to be an all or nothing event, the per-exposure probability was reduced by 68% relative to the vaccine group, with a predicted per exposure risk of infection of 0.068 relative to 0.087 for controls or 0.159 for vaccine only (Table 2). Interestingly there was no evidence of seroconversion in exposed but protected animals in the M group despite repeated viral challenge, (Fig. 5). These data concord with previous studies in humans and macaques where repeat vaginal exposure to 500µg of recombinant gp140 failed to induce antibody responses [27, 28]. The observed lack of seroconversion contrasts to those reported in a small number of subjects from the CAPRISA 004 trial [29], but concords with that seen in the Partners PrEP study [30]. It is unclear if induced antibody responses in CAPRISA 004 were dependent upon limited replication that was insufficient to establish infection.

The V+M combination was the only group to show a statistical difference to the naïve controls and to the V only group. Thus any potential vaccine related enhancement in per exposure risk of infection was mitigated by the combination of V+M. Furthermore, there was a 63% reduction (OR 0.39) in the V+M group relative to M alone, although this did not reach statistical significance. Nevertheless, the Kaplan-Meier curves from Part 1 predict that 9.5

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challenges would be required to infect 50% of animals in group M, while too few animals (4/16) were infected in group V+M to reliably calculate the same estimation for the combination group. Larger studies would be needed to confirm or refute a difference between V+M and M groups. However the positive interaction between microbicide and vaccine appeared to have a sustained effect in Part 2 where animals were challenged in the absence of microbicide (interaction coefficient p=0.13). This long-term benefit may indicate a durable effect of vaccination in this group or reflect that repetitive challenge selects animals with higher or increasing resistance to infection over time. The only observable impact of protected viral exposure on vaccine induced immunity in the V+M group were increased cellular responses to Env relative to the V group after 6 repetitive challenges. The significance of this finding is unclear as the level of cellular responses were not predictive of resistance to infection. Nevertheless, this echoes studies with the rhesus CMV vector encoding SIV Gag, Rev-Tat-Nef and Env that protected 50% of animals from productive infection despite inducing equivalent cell mediated responses in protected and unprotected animals [31]. By contrast, antibody levels were too high to ascertain any boost effects of protected exposure in the V+M group. Irrespective of the mechanism, the persistent positive impact for the V+M combination is encouraging.

Previous studies in NHP have suggested potential benefit from combining T cell based vaccine approaches with vaginal microbicides, however sample sizes were small and the microbicide approaches used have yet to be tested in human efficacy studies [32, 33]. This study is the first to assess the potential benefit of combining a microbicide with a humoral vaccine. We believe our data suggest that a microbicide-vaccine combination might provide greater efficacy then either intervention alone. The observed benefits are likely to be improved with a vaccine that contains optimal B and T cell immunogens. Perhaps more importantly, these data indicate

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452 that provision of ARV-prophylaxis ameliorates the potential for vaccine associated increased risk
453 of infection. Although we assessed the efficacy of tenofovir gel, the protective effects would
454 likely be similar or higher with oral prophylaxis, where compliance levels may be more reliable
455 [34]. This has important implications given that oral pre-exposure prophylaxis could be adopted
456 as the baseline intervention for future HIV/AIDS vaccine trials. Our findings are
457 contemporaneous with plans to evaluate a similar regimen to RV144 in South Africans, a
458 partially effective vaccine thought to be mediated by humoral immunity in the absence of Tier-2
459 neutralization [35, 36]. This has important implications given that oral pre-exposure prophylaxis
460 could be adopted as the baseline intervention for future HIV/AIDS vaccine trials.

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Conflict of interest statement: The authors have declared that no conflict of interest exists.

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TABLES:

Table 1. Odds ratios for Group effects on probability of infection Parts 1 and 2

Table 1A: Odds ratio for group effects on the probability of infection in Part 1(ref=group C).

Group	OR	IC 95%	p-value
Intercept	0.22	[0.09;0.53]	<0.001
Time	0.94	[0.87;1.00]	0.077
Group V+M vs C	0.21	[0.06;0.71]	0.013
Group M vs C	0.55	[0.20;1.56]	0.263
Group V vs C	1.73	[0.56;5.32]	0.341

Table1B: Odds ratio for group effects on the probability of infection in Part 2 (ref=group V).

Group	OR	IC 95%	p-value
Intercept	0.38	[0.15;0.95]	0.039
Time	0.94	[0.87;1.01]	0.077
Group V+M vs V	0.12	[0.03;0.44]	0.001
Group M vs V	0.32	[0.11;0.98]	0.045
Group C vs V	0.58	[0.19;1.78]	0.341

Table 1C: Odds ratio for treatment type on the probability of infection in Parts 1 and 2 combined.

Group	OR	IC 95%	p-value
Intercept	0.23	[0.11;0.47]	<0.001
Time	0.95	[0.92;0.98]	0.004
Vaccine (vs. control)	1.17	[0.49;2.82]	0.720
Microbicide (vs. control)	0.46	[0.18;1.20]	0.113
V+M vs C	0.16	[0.04;0.65]	0.010
V+M vs V	0.14	[0.04;0.50]	0.002

Table 1D: Odds ratio for treatment type on the probability of infection in Parts 1 and 2 combined (discretized time).

Group	OR	IC 95%	p-value
Intercept	0.22	[0.09;0.57]	0.001
Time in phase B	0.35	[0.10;1.19]	0.093
Vaccine (vs. control)	0.97	[0.28;3.28]	0.958
Microbicide (vs. control)	0.26	[0.06;1.02]	0.054
V+M (vs. control)	0.09	[0.02;0.46]	0.004
V+M (vs. vaccine)	0.09	[0.02;0.45]	0.003

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Table 1E: Odds ratio for treatment type on the probability of infection in Parts 1 and 2 combined depending on time (interaction of order 2 vac*mic*time).

Group	OR	IC 95%	p-value
Intercept	0.17	[0.06;0.47]	0.001
Time in phase B	0.75	[0.14;4.09]	0.738
Vaccine (vs. control)	1.91	[0.39;9.28]	0.425
Microbicide (vs. control)	0.37	[0.09;1.59]	0.185
V+M phase A (vs. control)	0.11	[0.02;0.59]	0.010
V+M phase B (vs. control)	0.02	[0.001;0.22]	0.002

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500 **Table 2. Summary analysis of infection risk in Part 1**

Animal Group	Number of Protected/ Total number of animals	Percentage Protected animals	Number of challenges	Nb of challenges to infect 50% of animals (95% CI)	P-value vs group C (Mantel-Cox test) Overall p=0.006	Hazard ratio (95%IC) (Cox model for time to infection)	Baseline risk of infection
C	4/12	33%	12	2.0 (1.0 - Undetermined)	-	-	0.087
V	1/8	13%	12	2.0 (2.0 - Undetermined)	0.568	1.53 (0.55-4.26)	0.159
M	6/14	43%	12	9.5 (5.0 - Undetermined)	0.272	0.56 (0.21-1.49)	0.068

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Table 3. Pharmacokinetics of vaginally applied 1% Tenofovir gel

TDF Tissue	Cmax (ng/g)	Tmax (h)	AUC0-12 (ngxh/g)
Serum	349 ± 196	1	793 ± 388
Vagina	151533 ± 89312	1	877034 ± 133061
Exocervix	286960 ± 169805	1	756546 ± 277502
Endocervix	39472 ± 29670	1	98139 ± 36024
Uterus	14449 ± 6068	1	38967 ± 13847
TDF-DP			
Tissue	Cmax (ng/g)	Tmax (h)	AUC0-12 (ngxh/g)
Vagina	94 ± 4	4	668 ± 142
Exocervix	64 ± 16	4	883 ± 571
Endocervix	<10	-	-
Uterus	<10	-	-

Foot Note Table 3: Pharmacokinetics of tenofovir (TDF) and tenofovir-diphosphate (TDF-DP) in female genital tract tissues after intravaginal administration of 1% tenofovir gel (mean ± SD). Tissues were sampled at necropsy (2 animals per time point) which were performed at 0, 1, 4 and 12 hours after gel administration. Tenofovir was quantified in serum by HPLC-MS/MS. Lower limit of quantification: 10 ng/g in vaginal, Exocervix and uterus and 25 ng/g in Endocervix. Tenofovir-diphosphate was quantified in serum by HPLC-MS/MS. Lower limit of quantification: 10 ng

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674 ## Figure Legends:

675 **Fig 1: Fig 1. Vaccination and challenge schedule with longitudinal serum antibody**
676 **responses in V+M group pre-challenge. (A)** Schematic representation of vaccination and
677 challenge schedules V – vaccine alone, V+M – vaccine + microbicide, M- microbicide alone, C-

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control Part 1 & Part 2. Green vertical arrows indicate intranasal vaccinations with R848 at weeks 0,4 & 8, red vertical arrows intramuscular vaccinations at weeks 16 &28 with MF59. Blue vertical arrows indicate 12 challenges in the absence of microbicide (weeks 39-59), black vertical arrows 12 challenges in the presence of microbicide (weeks 60-80)). (**B)** SF162 serum IgG responses in V+M group, SF162 serum IgA in (**C)** TV-1 serum IgG responses in (**D)** and serum IgA TV-1 responses in E all pre-challenge

Fig 2: Vaginal binding antibody responses for V+M group only post immunizations, prior to challenge. Antibody responses measured in vaginal secretion samples collected post – intranasal immunization (post IN at week 12) and after intramuscular immunizations (post IM) at week 34 prior to challenge. SF162 and TV-1 specific IgG antibodies are indicated as black symbols, red symbols show SF162 and TV-1 specific IgA responses (ng/ml). Lines indicate mean and +/- SEM for each group

Fig 3: Mapping of serum IgG binding to gp120 linear epitopes by peptide microarray: (A) & (B) Representative gp120 binding plots in serum of an immunized animal binding to either consensus gp120 sequences or vaccine strain gp120 at peak serum IgG response (2 weeks post last immunization). Numbers on X-axis are peptide numbers in the array library. Y-axis are signal intensity values (baseline subtracted). Different color bars represent different strains/clades as indicated. Variable –V and constant - C domains of gp120 are labelled in each panel**. (C)** Binding intensity to each epitope identified for the animals. The definition of each epitope as the range of peptide numbers in the array library is listed under each epitope. Color coding highlights higher intensity in darker red. Pie chart shows average percentage of binding to each epitope in the total. Each pie slice represents the mean values of all animals for maximum binding to the specified epitope/sum of maximum binding of all epitopes

Fig 4: Neutralizing antibody titers groups V+M and V: (A) & **(B)** show kinetics of neutralizing response after each immunization for the V+M group. (**C)** V - group. Sera were tested in TZMbl assay against pseudotype viruses (PSV) SF162 or MW965.26. Dotted lines represent vaccinations at weeks 0, 4, 8 (intranasal immunizations with R848), 16 and 28 weeks (intramuscular immunizations with MF59). **(D)** Shows neutralizing antibody titers of NHP of

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group V+M 30 weeks after the first immunization against a panel of PSVs of Tier 1 and Tier 2 tested in TZMbl assay. Solid horizontal lines in panel D indicate mean neutralizing titers

Fig 5: Time to event survival analysis Kaplan-Meier curves and infection of macaques determined by RT-PCR in plasma; V – vaccine alone, V+M – vaccine + microbicide, M- microbicide alone, C- control; (**A)** Kaplan-Meier plots of animals confirmed infected by RT-PCR. Red – Control animals, Purple – V group, Blue – V+M group, green – M group; (**B)** Plasma viral load of individual macaques during challenge phases; vertical dotted lines-date of challenges; horizontal lines – limit of quantification 300 copies /mL.

Fig 6. : Vaccine induced binding antibodies to SF162 in serum: V – vaccine alone, V+M – vaccine + microbicide, M- microbicide alone, C- control. (**A**) Serum response antibodies as ng/ml pre-challenge at week 34. (**B**) post-6 challenges. (**C)** post-9 challenges, (**D)** post-12 challenges. Colors are indicative of the time infection was detected by plasma viremia: Red during the first 6 challenges (till week 9 post-challenge), blue during challenges 7 to 12 (weeks 11 to 21 post-challenge), black during challenges 13 to 24 (from week 22, in absence of microbicide) and empty symbol for animals which did not show sign of infection.

Fig 7: Neutralizing antibody titres in serum against SF162: Sera were analyzed by TZMbl assay, at 12 (post IN) and 34 weeks (post IM) immunization **(A)**. Pre-challenge **(B)** post-6 challenges **(C)**, post-9 challenges **(D)** and post-12challenges (**E**). Each symbol represents one animal, colors are indicative of the time infection was detected by plasma viremia: Red during the first 6 challenges (up to 9 weeks post-challenge), blue, challenges 7 to 12 (weeks 11 to 21 post-challenge), black challenges 13 to 24 and empty symbols for animals which did not get infected. P = n.s. between V and V+M at each time-point.

Fig 8. Neutralizing antibody responses against SF162 during challenge phases Part 1 & Part 2: TZMbl assay neutralization data 5 weeks before challenge (0 wk), 9 weeks, 17 weeks and 21 weeks after the first challenge with microbicide, and one week after the 12 challenges without microbicide (week 42) and 5 weeks thereafter (week 47). Each symbol represents one animal. Colors are indicative of the time infection was detected by plasma viremia: Red during the first 6 challenges (till week 9 post-challenge), blue during challenges 7 to 12 (weeks 11 to 21

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post-challenge), black during challenges 13 to 24 (from week 22, in absence of microbicide) and empty symbols for animals which did not get infected.. The mean is indicated for each group with a solid line. V+M vs. V $p< 0.0001$; Mann-Whitney

Fig 9. T-cell responses measured by Elispot assay pre and post challenge: gp120 specific T cell Elispot responses as spot forming cells (SFU)/10^6 PBMC pre-challenge, **(A) & (B),** post 6 challenges **(C) & (D).** SIV-gag specific T cell Elispot responses pre-challenge **(E) & (F)** and post 6 challenges in **(G) & (H).** Red symbols indicate infection during the first 6 challenges, blue during challenges 7 to 12 (weeks 11 to 21 post-challenge), black during challenges 13 to 24, empty symbols for animals which did not get infected. Solid lines indicate means and p-values where relevant following statistical analysis.

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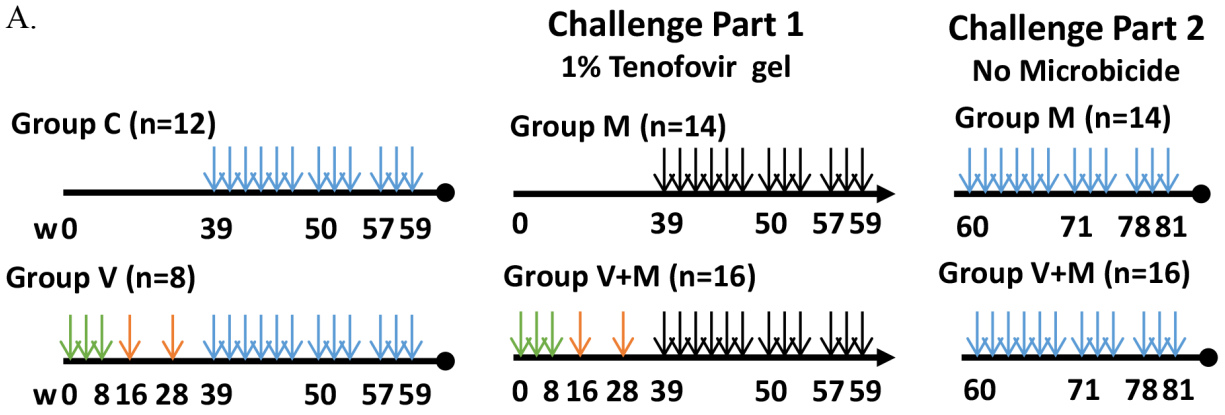

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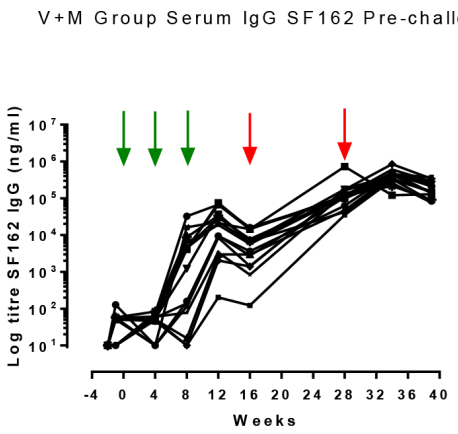

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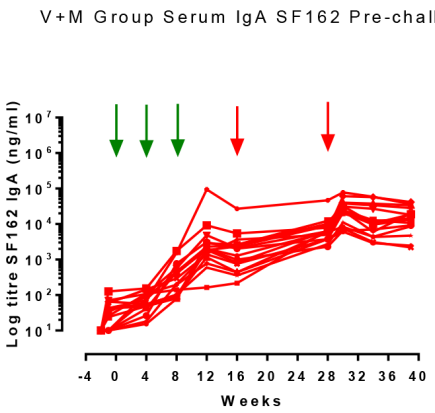

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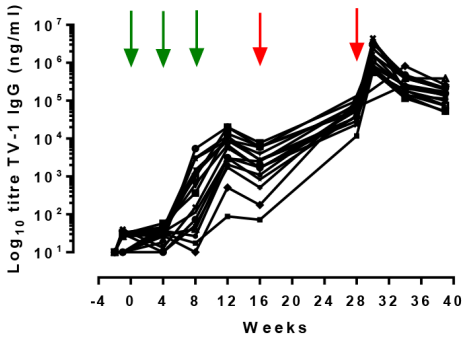

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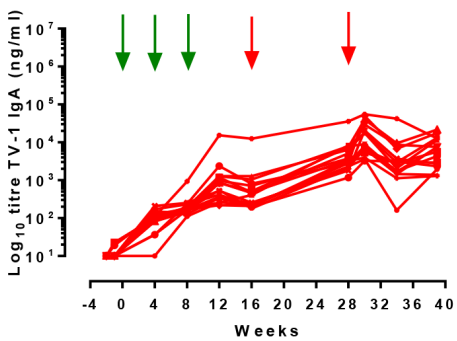

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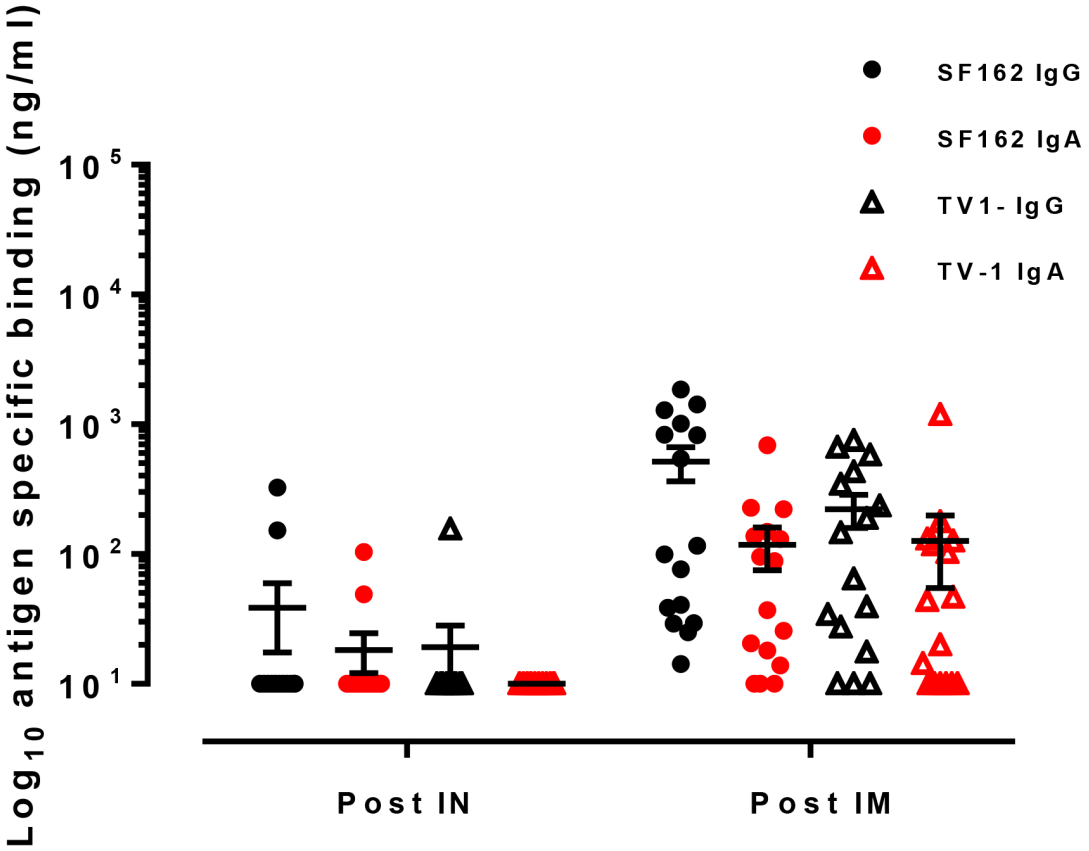

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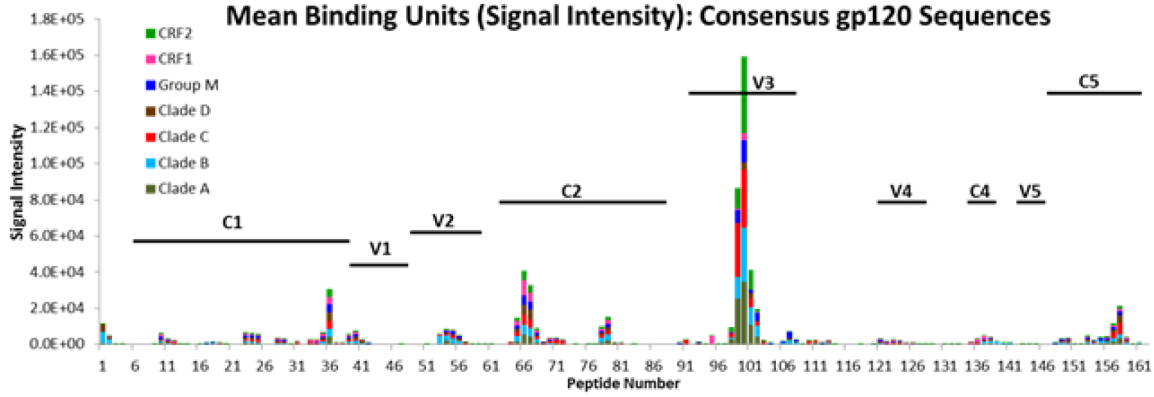

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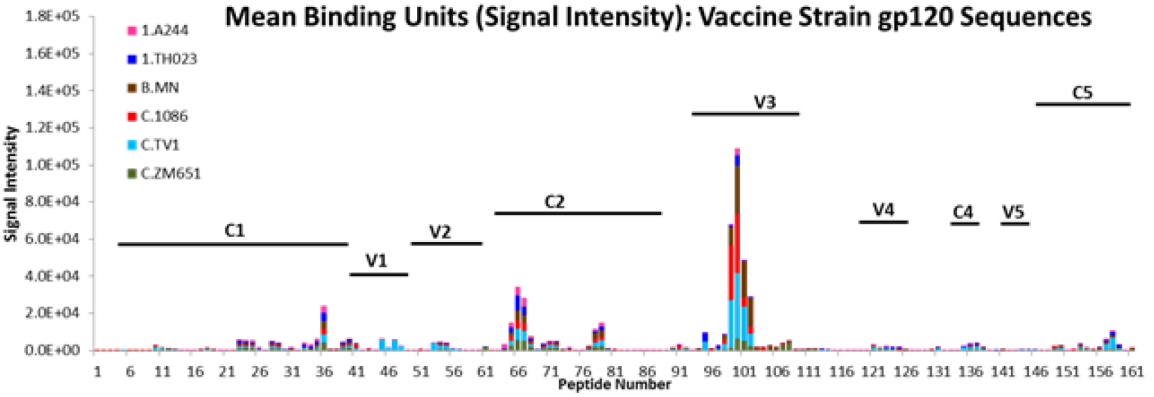

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23566	562	1,251	5,410	8,200	11,115	1,343	54,475	6,082	23077
23571	1,997	12,610	819	3,615	8,566	2,715	56,100	13,192	11790
23572	1,698	3,255	169	2,988	4,223	1,455	49,248	2,794	8323
23581	394	4,651	7,628	1,825	6,948	3,529	31,643	3,206	3557
23592	16,330	1,648	26,785	2,959	5,788	2,507	31,899	7,960	14373
23682	2,086	1,635	22,439	15,123	5,929	2,098	60,280	21,149	29259
23712	7,506	24,333	1,715	9,978	13,194	8,456	36,350	8,760	23296
28137	2,606	12,914	18,019	27,949	21,886	8,722	56,154	10,078	20994
30265	6,200	3,136	11,220	5,878	8,987	4,038	48,659	5,535	7136
30087	1	2,492	13,050	678	6,422	703	33,543	4,756	2046
30207	1,117	2,979	4,236	4,015	3,232	4,009	48,786	2,210	4982
30213	1	1,471	10,346	1,671	2,666	5,269	63,211	3,190	3767
30253	1	628	5,183	2,412	9,117	2,999	46,190	7,645	5202
30554	1	1,125	3,845	1,510	1,248	3,260	15,119	5,937	7985
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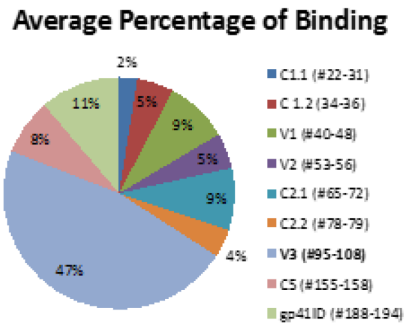

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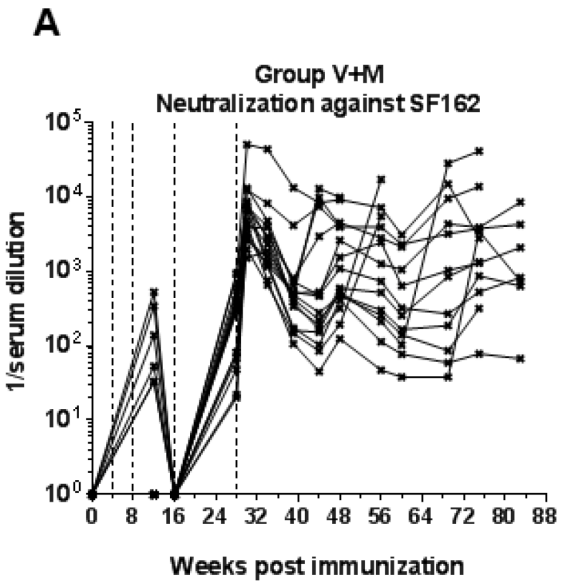

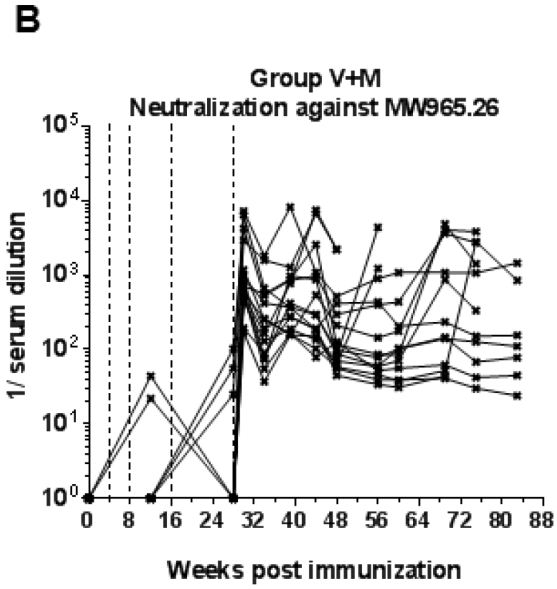

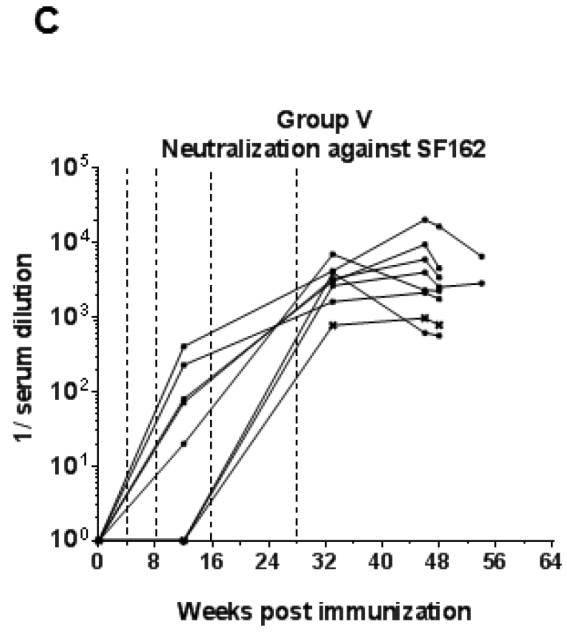

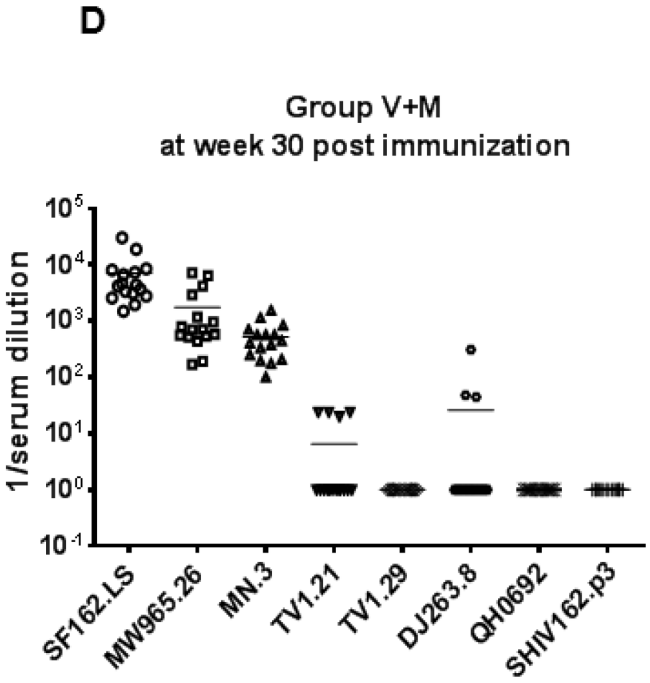

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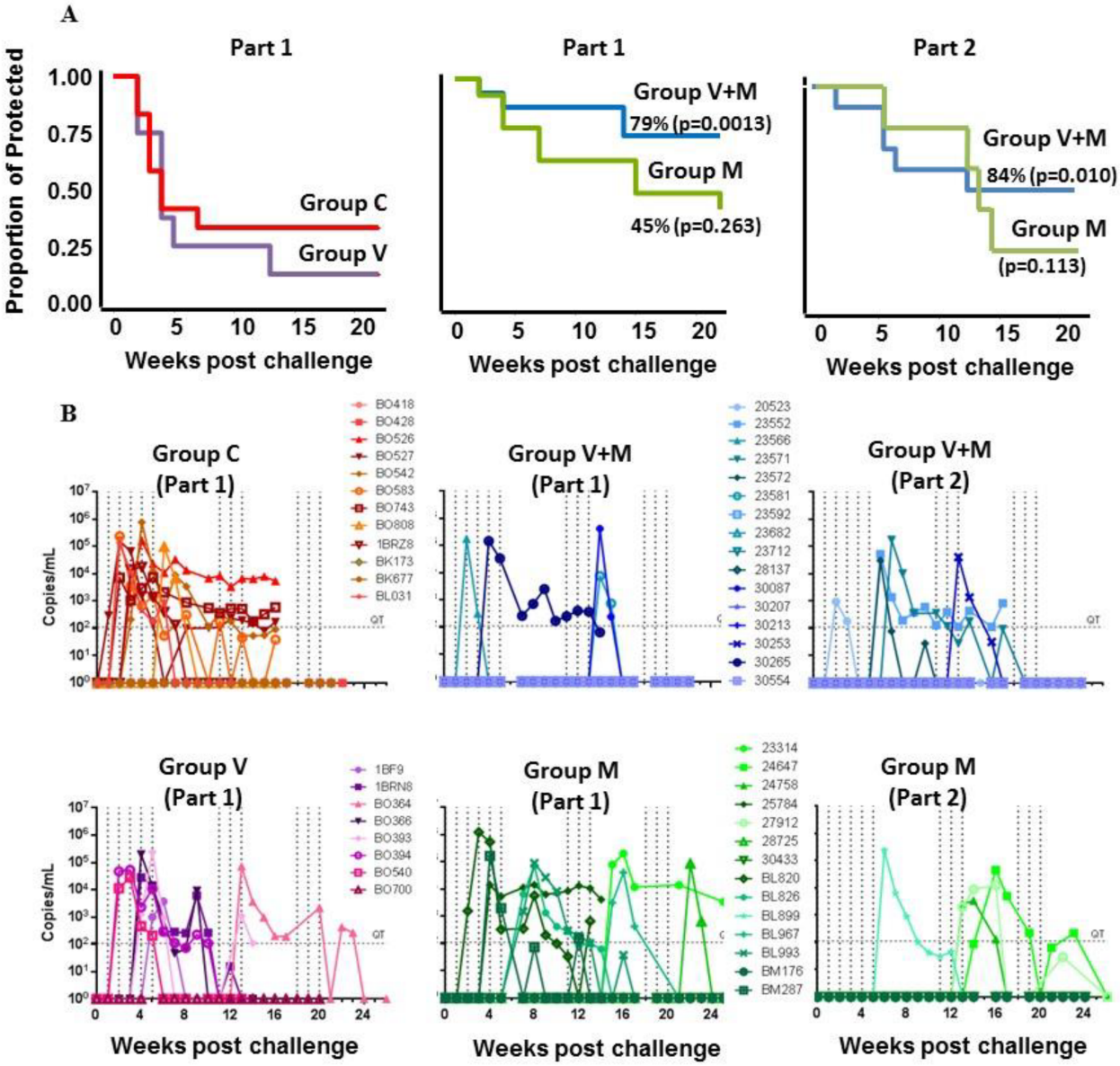

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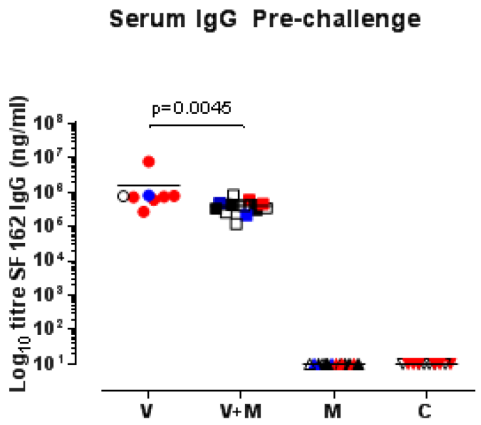

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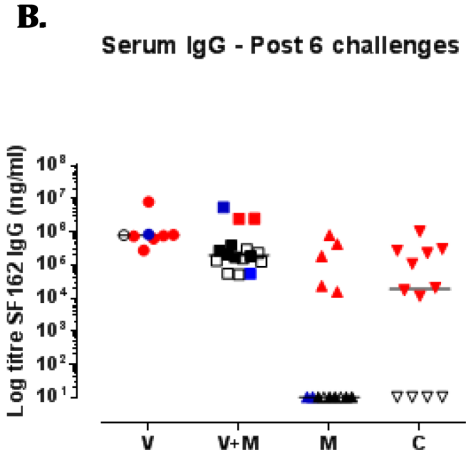

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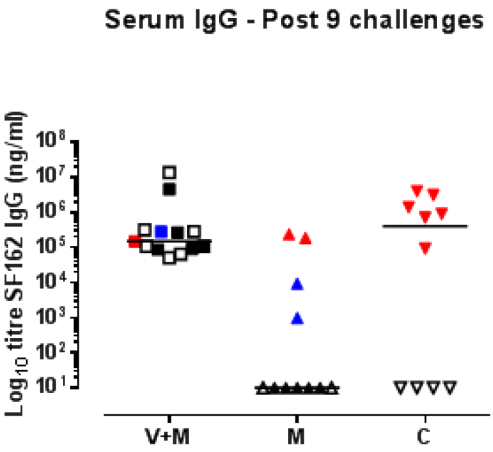

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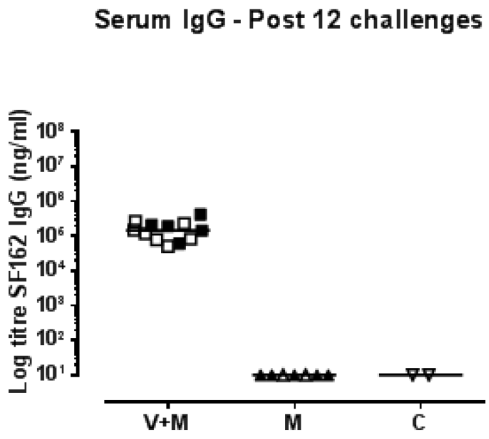

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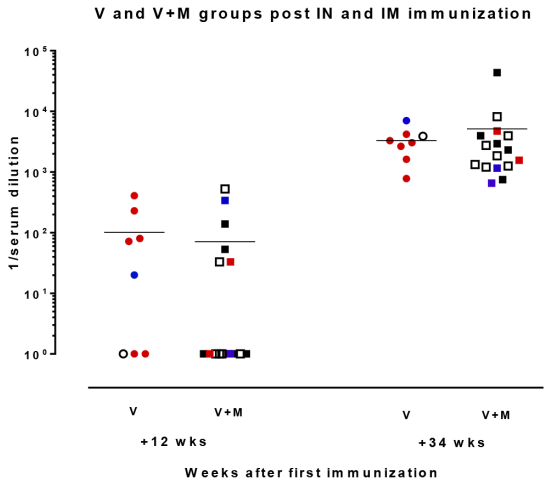

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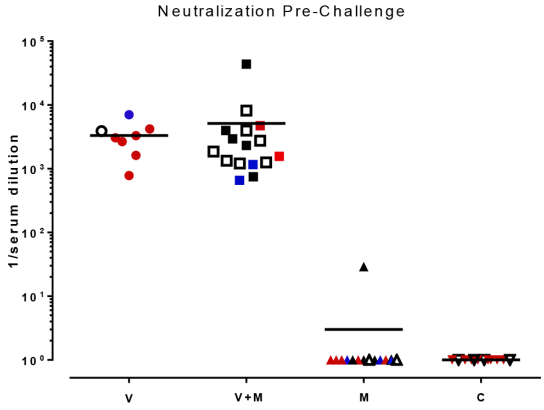

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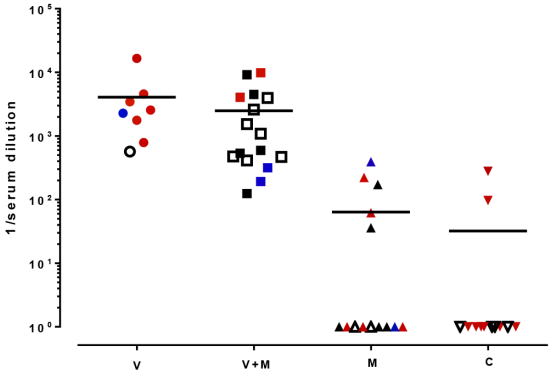

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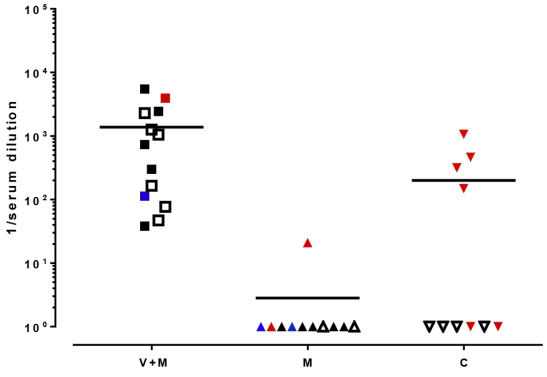

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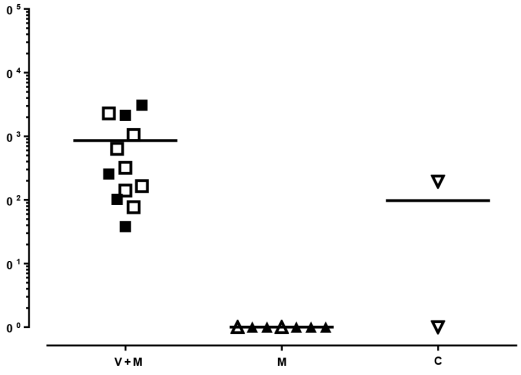

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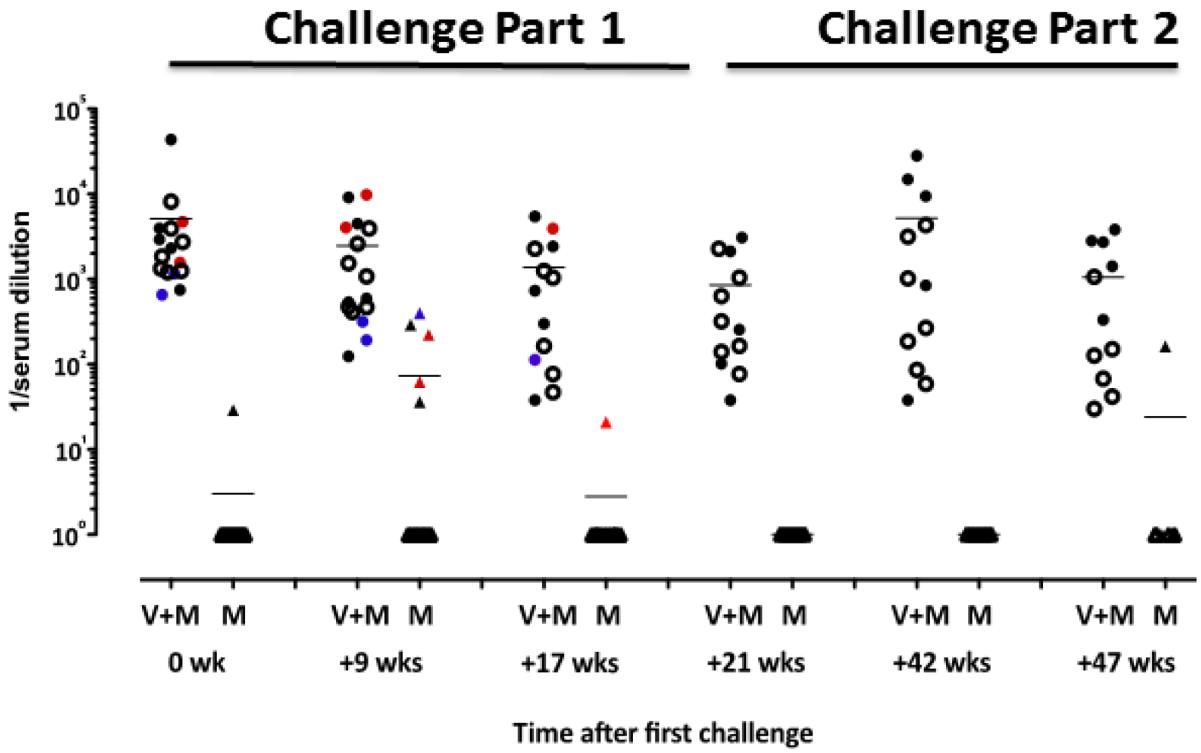

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